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Review

A dual role for the anti-apoptotic Bcl-2 protein in cancer: Mitochondria versus endoplasmic reticulum[☆]Haidar Akl^{*}, Tamara Vervloessem, Santeri Kiviluoto, Mart Bittremieux, Jan B. Parys, Humbert De Smedt, Geert Bultynck^{*}

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ABSTRACT

Anti-apoptotic Bcl-2 contributes to cancer formation and progression by promoting the survival of altered cells. Hence, it is a prime target for novel specific anti-cancer therapeutics. In addition to its canonical anti-apoptotic role, Bcl-2 has an inhibitory effect on cell-cycle progression. Bcl-2 acts at two different intracellular compartments, the mitochondria and the endoplasmic reticulum (ER). At the mitochondria, Bcl-2 via its hydrophobic cleft scaffolds the Bcl-2-homology (BH) domain 3 (BH3) of pro-apoptotic Bcl-2-family members. Small molecules (like BH3 mimetics) can disrupt this interaction, resulting in apoptotic cell death in cancer cells. At the ER, Bcl-2 modulates Ca²⁺ signaling, thereby promoting proliferation while increasing resistance to apoptosis. Bcl-2 at the ER acts via its N-terminal BH4 domain, which directly binds and inhibits the inositol 1,4,5-trisphosphate receptor (IP₃R), the main intracellular Ca²⁺-release channel. Tools targeting the BH4 domain of Bcl-2 reverse Bcl-2's inhibitory action on IP₃R and trigger pro-apoptotic Ca²⁺ signaling in cancer B-cells, including chronic lymphocytic leukemia (CLL) cells and diffuse large B-cell lymphoma (DLBCL) cells. The sensitivity of DLBCL cells to BH4-domain targeting tools strongly correlated with the expression levels of the IP₃R2 channel, the IP₃R isoform with the highest affinity for IP₃. Interestingly, bio-informatic analysis of a database of primary CLL patient cells also revealed a transcriptional upregulation of IP₃R2. Finally, this review proposes a model, in which cancer cell survival depends on Bcl-2 at the mitochondria and/or the ER. This dependence likely will have an impact on their responses to BH3-mimetic drugs and BH4-domain targeting tools. This article is part of a Special Issue entitled: Calcium signaling in health and disease. Guest Editors: Geert Bultynck, Jacques Haiech, Claus W. Heizmann, Joachim Krebs, and Marc Moreau.

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Abbreviations: ATP, adenosine triphosphate; BI-1, Bax inhibitor-1; BH, Bcl-2-homology; CN, calcineurin; CaM, calmodulin; CLL, chronic lymphocytic leukemia; JNK1, c-Jun N-terminal kinase 1; DLBCL, diffuse large B-cell lymphoma; DARPP-32, dopamine- and cAMP-regulated phosphoprotein of 32 kDa; ER, endoplasmic reticulum; ERK, extracellular-signal-regulated kinases; GM-CSF, granulocyte-macrophage colony-stimulating factor; IP₃R, inositol 1,4,5-trisphosphate receptor; IRE1α, inositol-requiring enzyme-1 alpha; IL-2 and IL-3, interleukin (IL)-2 and -3; IICR, IP₃-induced Ca²⁺ release; MAMs, mitochondria-associated ER membranes; MOMP, mitochondrial outer membrane permeabilization; NFAT, nuclear factor of activated T-cells; PTEN, phosphatase and tensin homolog; PI3K, phosphatidylinositol 4,5-bisphosphate 3-kinase; Akt/PKB, phosphoinositide-dependent serine-threonine protein kinase/protein kinase B; PKA, protein kinase A; SERCA, sarco/endoplasmic reticulum Ca²⁺-ATPase; TAT-IDP^S, stabilized TAT-IP₃R-derived peptide; Orai1, STIM1-mediated Ca²⁺-release-activated Ca²⁺-channel protein 1; STIM1, stromal interaction molecule 1; UPR, unfolded protein response; VDAC, voltage-dependent anion channel

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1. Introduction

Bcl-2, the founding member of the Bcl-2 family, derives its name from *B-cell lymphoma 2*. It was initially described in 14;18 chromosome translocations, which are a characteristic of follicular lymphoma, the most common lymphoma in humans [1,2]. The Bcl-2-protein family regulates cell death and proliferation [3,4], two processes dysregulated during oncogenic transformation. Thus, it is not surprising to find an upregulation of Bcl-2 expression in many cancers, including chronic lymphocytic leukemia (CLL) and diffuse large B-cell lymphoma (DLBCL) [5,6].

Intracellular Ca²⁺ signals originating from the endoplasmic reticulum (ER), the main intracellular Ca²⁺-storage organelle, also control cell death and proliferation [7]. In particular, the close proximity of the ER and the mitochondria allows a swift and prominent accumulation of Ca²⁺ into the mitochondria, not only in response to physiological agonists but also in response to apoptotic stimuli that promote Ca²⁺ release from the ER [8–10]. Moreover, intracellular Ca²⁺ elevation induces calcineurin (CN)-dependent dephosphorylation and activation of Bad, a pro-apoptotic Bcl-2 protein, leading to Bcl-2 antagonism and mitochondrial outer membrane permeabilization (MOMP) [11]. In

normal conditions, inactive Bad is highly phosphorylated and binds to 14-3-3 scaffold proteins and thus cannot interact with Bcl-2 or Bcl-XL [12]. The inositol 1,4,5-trisphosphate receptor (IP₃R) is the main intracellular Ca²⁺-release channel located in the ER. Importantly, a subfraction of the IP₃R is located in a microdomain of the ER, namely the mitochondria-associated ER membranes (MAMs) [10,13–15]. The magnitude and the duration of Ca²⁺ signals derived from the IP₃R are decisive for the cell fate, for which small Ca²⁺ oscillations promote mitochondrial bioenergetics and cellular proliferation, while large Ca²⁺ transients cause MOMP and subsequent apoptosis [16,17]. It is therefore not surprising that Bcl-2 proteins also act at the level of the ER as critical regulators of the IP₃R channels, thereby assuring Ca²⁺-dependent cell proliferation while protecting them from apoptosis. In this review, the key determinants of Bcl-2's action at the mitochondria and at the ER are discussed, thereby illustrating two different strategies that cancer cells can use to promote survival.

2. The canonical anti-apoptotic role of Bcl-2 at the mitochondria

The Bcl-2 family of proteins is essentially studied for its role in apoptosis [18–21]. Apoptosis is a programmed cell death morphologically characterized by membrane blebbing, cell shrinkage, chromatin condensation and chromosomal DNA fragmentation [22]. Under physiological conditions, apoptosis is a non-immunogenic cell death, because the produced apoptotic bodies are removed by the phagocytic cells before the contents of the dying cell can induce an inflammatory reaction [23]. There are two distinct signaling pathways that lead to apoptosis: (i) the extrinsic or extracellular pathway induced, for example, by an infection that activates a receptor-mediated process, like the tumor necrosis factor receptor (TNFR) [24] or the Fas receptor (FasR) [25] and (ii) the intrinsic or mitochondria-mediated pathway [26–28]. The intrinsic apoptosis signaling pathway is operated by the Bcl-2-family members, which are generally divided into three categories (anti-apoptotic proteins, pro-apoptotic executioners and pro-apoptotic BH3-only proteins) based on their intracellular function and sequence homology [29]. The anti-apoptotic Bcl-2 proteins like Bcl-2, Bcl-w, Mcl-1, Bfl-1 and Bcl-XL which contain all four Bcl-2-homology (BH) domains 1–4 (BH1–4), can interact with both pro-apoptotic categories, the multi-domain executioner proteins Bax and Bak and the BH3-only proteins. The latter consist of two groups: the activator BH3-only proteins, which can directly activate the executioners Bax and Bak, like Puma, Bim, and Bid and the sensitizer BH3-only proteins, like Bad, Bik, Noxa and Bmf which de-repress Bcl-2's anti-apoptotic function but cannot directly activate Bax and Bak [28] (Table 1). Mechanistically, anti-apoptotic family members prevent death by binding and sequestering the BH3 domains of activator BH3-only proteins and preventing their interaction with Bax/Bak [30,31]. The sensitizers BH3-only proteins induce Bax/Bak oligomerization indirectly by binding anti-apoptotic proteins and thereby displacing activator BH3-only proteins [31]. Very recently, a selective role for Bid and Bim as activator BH3-only proteins has been discovered: Bid preferentially switches on Bak, while Bim preferentially switches on Bax [32] (Fig. 1).

After their oligomerization induced by the BH3-only proteins, Bax and Bak directly cause MOMP, a critical step during apoptosis [26,27,

33]. Cytochrome c is released after MOMP leading to the activation of caspases and the subsequent progression toward dismantling of the cell [27]. Practically, post-MOMP, mitochondria are impaired in their ability to generate adenosine triphosphate (ATP) and cannot maintain cellular homeostasis [27,34,35]. Therefore, MOMP is considered as the point-of-no-return in mitochondrial apoptosis [21].

The balance between pro-survival and pro-death Bcl-2 proteins is a major factor in determining whether or not cells undergo apoptosis in response to cell stress. Anti-apoptotic Bcl-2 members can via their hydrophobic cleft composed by their BH1, 2 and 3 domains, bind and sequester the pro-apoptotic Bcl-2-family members (Fig. 1). Via this hydrophobic cleft, the anti-apoptotic Bcl-2 proteins can also interact with other apoptosis regulators that are not members of the Bcl-2-protein family, like p53 [28]. Beyond these interactions, Bcl-2 can also interact with several non-Bcl-2-family proteins via its N-terminal BH4 domain. It can e.g. target the serine/threonine protein kinase Raf-1 to mitochondrial membranes, allowing it to phosphorylate Bad [36]. Moreover, Bcl-2 can sequester active NFAT and subsequently block the nuclear factor of activated T-cells (NFAT)-signaling pathway [37].

3. ER-to-mitochondria Ca²⁺ transfer determines cell fate

3.1. Ca²⁺ is a key factor in mitochondria-based cell fate

Mitochondrial homeostasis is essential for the regulation of bioenergetics, cell proliferation, cell death and autophagy, a survival mechanism that involves cell degradation of unnecessary cellular components in order to produce energy [7,9,17,38–41]. In fact, Ca²⁺ is a key regulator of mitochondrial homeostasis and consequently has a pivotal role in determining cell fate [41]. While moderate Ca²⁺ levels are essential for normal mitochondrial activities, mitochondrial overload of Ca²⁺ is detrimental for the morphology of this energetic organelle. High mitochondrial Ca²⁺ levels cause mitochondrial depolarization, thereby opening the mitochondrial permeability transition pore (mPTP), whose molecular nature has recently been identified as the c subunit of the F₀F₁-ATPase [42–44]. mPTP opening leads to mitochondrial swelling and MOMP, the point-of-no-return for apoptosis induction by triggering the release of cytochrome c [44]. Recently, mitochondrial Ca²⁺ overload has also been implicated in mitophagy, the selective degradation of damaged mitochondria through autophagy (as reviewed in [14]). In contrast, too low mitochondrial Ca²⁺ levels reduce ATP production, thereby leading to the activation of AMP-activated kinase and the induction of autophagy [45].

3.2. The IP₃R is an ER Ca²⁺ channel playing a central role in cell-fate decision

The Ca²⁺ transfer from the ER to the mitochondria involves the IP₃R and the voltage-dependent anion channel (VDAC) linked through the 75-kDa glucose-regulated protein (GRP75). This molecular bridge, as well as the presence of other ER-mitochondria tethers like the mitochondria-shaping proteins mitofusin-1 and -2, helps to establish ER/mitochondria contacts at the MAMs [9,10,46,47]. The ER-to-mitochondria Ca²⁺ transfer depends on the filling state of the ER Ca²⁺ stores as well as on the Ca²⁺-flux properties of the IP₃R [48]. In basal conditions, constitutive low-level IP₃R-mediated Ca²⁺ release is essential to fuel mitochondria with Ca²⁺ necessary for the activity of mitochondrial enzymes like pyruvate-, α -ketoglutarate- and isocitrate-dehydrogenases [45,49,50]. Basal Ca²⁺ oscillations are responsible for the production of ATP and nicotinamide adenine dinucleotide phosphate (NADPH). Ca²⁺ also indirectly fuels bioenergetics by stimulating substrate transporters such as the ARALAR/AGC1-malate aspartate shuttle [51,52]. Previous data have shown that sensitizing the IP₃R to basal IP₃ levels, as via interaction with Bcl-XL, promotes bioenergetics and cell survival [53,54]. Furthermore, in normal cells, the inhibition of the IP₃R-dependent Ca²⁺ fluxes induces basal autophagy as a pro-survival

Table 1

The Bcl-2 family members are divided into three categories based on their intracellular function and sequence homology.

Anti-apoptotic	Pro-apoptotic		
All four BH domains (BH1–4)	Multi-domain (BH1–3)	BH3-only proteins	
Sequester their pro-apoptotic counterparts	Executioners	Activators	Sensitizers
Bcl-2, Bcl-XL, Bcl-w, Bfl-1 and Mcl-1	Bax and Bak	Bim and Bid	Bad, Bik, Noxa and Bmf

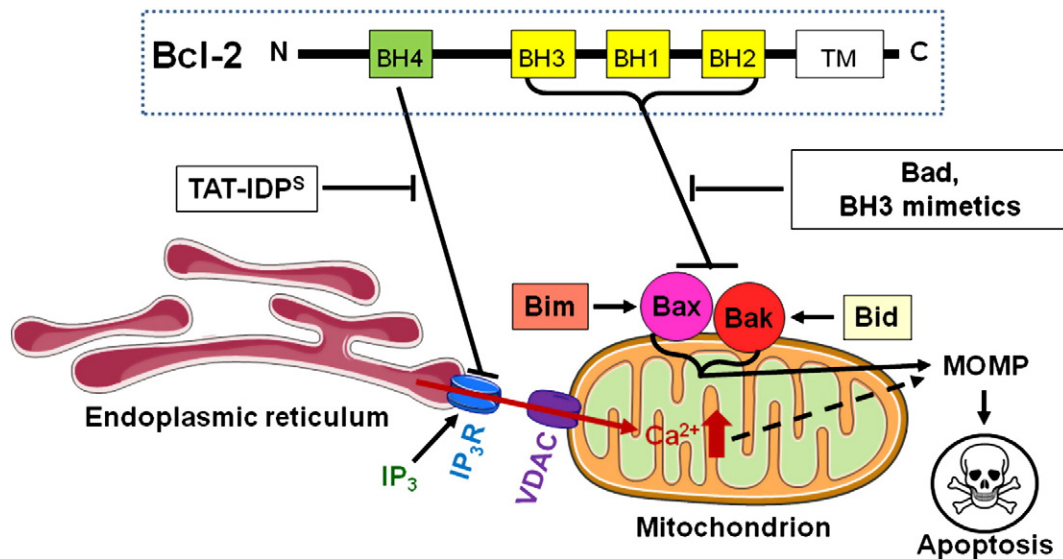


Fig. 1. Dual role of anti-apoptotic Bcl-2 at both the mitochondria and the ER. At the mitochondria, Bcl-2 binds Bax/Bak via its hydrophobic groove composed of the BH1, 2 and 3 (yellow boxes), preventing their oligomerization and inhibiting Bax/Bak-pore formation. This can be counteracted by the action of sensitizer BH3-only domains (Bad) or mimicking molecules (BH3 mimetics). Bid preferentially activates Bak and Bim preferentially activates Bax, leading to Bax/Bak-dependent pore formation followed by MOMP, the point-of-no-return in apoptosis. At the ER, Bcl-2 interacts with the IP₃R via its N-terminal BH4 domain (green box), thereby inhibiting its Ca²⁺-flux properties. The IP₃R mimetics (like TAT-IDP⁵) can relieve the inhibition of IP₃Rs by Bcl-2, leading to increased Ca²⁺ signaling in the presence of constitutive IP₃ production in cancer cells. The released Ca²⁺ could be gated into the mitochondria via a protein complex including the VDAC channel, leading to mitochondrial Ca²⁺ overload, indirectly (dashed line) causing apoptosis. In anti-cancer therapy Bcl-2 can be targeted at both sites, and both strategies are expected to be complementary.

response [40,45]. Many studies have shown that IP₃R deficiency protects cells from cell death [56–61]. Some of these studies were based on IP₃R1-deficient B- and T-lymphocytes that are resistant to apoptosis induced by dexamethasone, T-/B-cell-receptor stimulation, ionizing radiation, Fas, and staurosporine [56,57]. Moreover, specific inhibition of Ca²⁺ release from the IP₃R strongly blocks lymphocyte apoptosis [59]. Furthermore, in aging oocytes, the Ca²⁺ leak from the IP₃R1 channel domain after truncation by caspase 3 acts as a feed-forward mechanism to divert the cell into apoptosis [60]. The anti-apoptotic phosphoinositide-dependent serine–threonine protein kinase/protein kinase B (Akt/PKB) has been shown to phosphorylate IP₃R3 and IP₃R1, thereby reducing ER Ca²⁺ release and protecting cells from apoptosis [61,62]. Recently, it has been shown that the tumor suppressor phosphatase and tensin homolog (PTEN) can interact with the IP₃R3 at the ER, and that this correlates with a decrease in Akt/PKB-mediated phosphorylation of the receptor and a subsequent reduction of Ca²⁺ release via the IP₃R3 [63]. Moreover, several members of the transmembrane Bax-inhibitor motif (TMBIM) family are located at the ER and impact survival and apoptosis by directly targeting IP₃Rs [64]. For instance, BI-1 directly binds and stimulates IP₃R channels [65]. Interestingly, the stimulatory effect of BI-1 on autophagic flux has been linked to its ability to promote Ca²⁺ signaling and thus to lower steady-state ER Ca²⁺ levels [55]. However, BI-1 has also been reported to suppress autophagy by directly targeting and inhibiting the ER-stress sensor, inositol-requiring enzyme 1 α (IRE1 α) [66,67]. Cells lacking BI-1 display IRE1 α -mediated c-Jun N-terminal kinase 1 (JNK1) activation [67], which disrupts the autophagy-inhibiting Bcl-2/Beclin 1 complex and thus activates autophagy [68]. Another common regulator of IRE1 α and IP₃Rs is glutamate receptor ionotropic N-methyl D-aspartate-associated protein (GRINA, or TMBIM3), which is upregulated in a protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK)/activating transcription factor 4 (ATF4)-dependent manner during ER stress and which suppresses IP₃R-mediated Ca²⁺ release as an adaptive protective response [69]. However, excessive ER stress has been associated with a prolonged and detrimental attenuation of the IP₃R1 function by disrupting the complex between IP₃R1 and 78 kDa glucose-regulated protein (GRP78) that is essential for IP₃R1-subunit assembly into functional channels [70]. These mechanisms underpin the complex role of

intracellular Ca²⁺ signaling mediated by IP₃Rs in cell survival and cell death in normal and stress conditions [71].

IP₃-induced Ca²⁺ release (IICR) can also control cell proliferation independently of its mitochondrial function. Several transcription factors regulating growth factor expression are known to be regulated by IICR [72,73]. One typical example is NFAT, that is involved in the transcriptional regulation by cell surface receptors, cytokines (e.g. interleukin (IL)-2), as well as other transcription factors in lymphocytes. NFAT proteins are dephosphorylated by CN and subsequently translocated from the cytoplasm to the nucleus. CN is activated by the Ca²⁺ mobilization initiated by the IP₃R, followed by Ca²⁺ influx via the stromal interaction molecule 1 STIM1-mediated Ca²⁺-release-activated Ca²⁺-channel protein 1 (Orai1) [74–78] (Fig. 2). Interestingly, it has been shown that CN activation protected the leukemic cells from apoptosis induced by interleukin-2 (IL-2) withdrawal in vitro [72] and favored leukemia progression in vivo [79].

Due to its critical role in intracellular Ca²⁺ dynamics, the IP₃Rs are tightly regulated by many proteins, as previously reviewed in [7,41,80,81]. Dysregulation of the IP₃R expression, function or interactome can result either in an escape from cell death as in some types of cancer, or in exaggerated cell death as in neurodegenerative diseases [80]. Among this vast group of IP₃R-interacting proteins, we will focus in this review on the role of Bcl-2 in the control of IP₃Rs during cancerogenesis.

3.3. IP₃R biology in cancer

Many oncoproteins and tumor suppressors act on the cellular Ca²⁺-signaling toolkit to control cell death and proliferation [82]. Moreover, it becomes increasingly clear that dysregulated Ca²⁺ homeostasis may contribute to the survival of malignant cells [82]. As such, the expression and function of different Ca²⁺-permeable channels and Ca²⁺ pumps (like IP₃Rs, transient receptor potential (TRP) channels, Orai1, sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA), plasma membrane Ca²⁺-ATPase (PMCA) and secretory pathway Ca²⁺-ATPase (SPCA)) have been reported to be altered in a plethora of cancers [83–85]. A growing number of studies now indicate that the function of IP₃Rs as signaling platforms is influenced by different oncoproteins, like Akt/

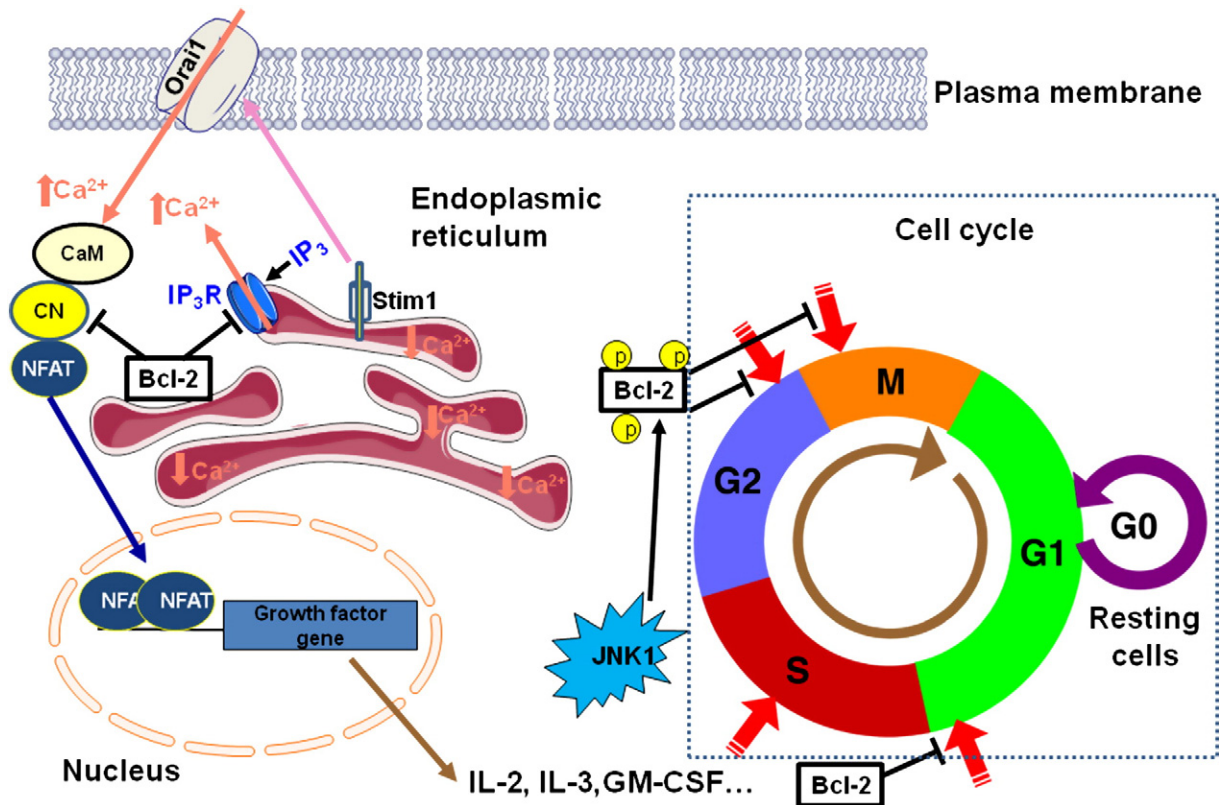


Fig. 2. Interaction between Bcl-2 and the cell cycle. Bcl-2 can directly or indirectly inhibit the proliferation of cells at different check points of the cell cycle (the check points of the cell cycle are represented by large red arrows). At the G₀/G₁ transition phase, Bcl-2 controls the expression pattern of p130, p27 and retinoblastoma protein (pRb) (not shown in the figure) to induce an arrest in the G₁ phase. Phosphorylation of Bcl-2 (Thr69, Ser70 and Ser87) by JNK1 leads to cell-cycle arrest at G₂/M. Bcl-2 interacts with and inhibits calcineurin (CN) and IP₃Rs. CN is activated by IP₃-induced Ca²⁺ release from the ER and subsequent Ca²⁺ entry via STIM1-gated Orai1 channels. This will result in an increase in cytosolic Ca²⁺ levels and the activation of Ca²⁺/calmodulin (CaM)-dependent phosphatase CN, which will dephosphorylate NFAT proteins. This activated NFAT will translocate to the nucleus, where it drives the transcription of growth factor genes, like IL-2, IL-3 and GM-CSF. As a consequence of the inhibitory effect of Bcl-2 on both CN and IP₃R, NFAT nuclear translocation, and IL-2, IL-3 and GM-CSF expression are impaired. In the absence of these growth factors, the DNA synthesis during the S phase of the cell cycle is affected.

PKB, anti-apoptotic Bcl-2-family members, BI-1, K-ras-induced actin-interacting protein (KRAP), and by tumor suppressors, like promyelocytic leukemia protein (PML) and Beclin 1 (as reviewed in [7]). These interactions and modifications may alter IP₃R-mediated Ca²⁺ signaling and provide cell survival despite the ongoing pro-apoptotic signaling due to oncogenic stress, to which these cells are exposed in cancer conditions [37]. The pathways by which cancer cells exploit altered IP₃R-mediated Ca²⁺ signaling to promote cell survival and/or to increase resistance to apoptosis, reveal important roles for Akt/PKB [62], PML [86] and Bcl-2 proteins [87] as inhibitory mechanisms that prevent toxic IP₃R-mediated Ca²⁺ signaling. These IP₃R-interacting proteins show a selective targeting of particular IP₃R isoforms [10,61,63,8]. Furthermore, there is growing evidence for a specific role of IP₃R isoforms in tumorigenesis, metastasis and resistance. For instance, a prominent role for IP₃R3 channels has been implicated in different types of cancer [89–92]. It has been shown that i) inhibition of IP₃R-mediated Ca²⁺ release by caffeine blocks migration of glioblastoma cells in different *in vitro* assays [89], ii) the elevation of IP₃R3 expression by estradiol is involved in the role of IICR in the growth of the MCF-7 breast cancer cell line [90], iii) the expression level of IP₃R3 in colon cancer cells is directly related to aggressiveness of the tumor resulting from increased resistance to apoptosis [91], and iv) the molecular interaction and functional coupling between IP₃R3 and BK_{Ca}, a voltage- and Ca²⁺-dependent K⁺ channel, stimulate breast cancer cell proliferation [92]. On the other hand, reduction in IP₃R1 mRNA levels was associated with the acquisition of cisplatin resistance in bladder cancer cells [93]. In fact, the suppression of IP₃R1 expression using small interfering RNA prevented apoptosis and resulted in decreased sensitivity to

cisplatin, while overexpression of IP₃R1 in resistant cells induced apoptosis and increased sensitivity to cisplatin [93].

4. Bcl-2 at the ER: protection against excessive IP₃R-mediated Ca²⁺ signaling in B-cell cancers

Different Bcl-2-family members have been described to be located at intracellular membranes other than the mitochondria, including the ER [94–97]. These Bcl-2-family members can affect different aspects of ER-related functions [98]. For instance, Bax/Bak proteins directly target the ER-stress sensor, IRE1 α , and are essential for the activation of IRE1 α and downstream unfolded protein response (UPR) signaling [99]. Also, Bcl-2 proteins have been implicated to recruit pro-apoptotic BH3-only proteins like Bim to the IRE1 α complex as an adaptive mechanism to sustain UPR signaling during ER stress [100].

Besides the direct regulation of UPR signaling, Bcl-2 proteins regulate ER Ca²⁺ homeostasis and dynamics, which also directly affect protein folding [39,101–103]. Over the years, strong evidence for the control of Ca²⁺ release from the ER by Bcl-2 has been accumulating, but different, not mutually exclusive mechanisms have been proposed. For instance, Bcl-2 has been reported to lower the steady-state ER Ca²⁺-store content, thereby preventing the occurrence of excessive pro-apoptotic Ca²⁺ signals and mitochondrial Ca²⁺ overload [97,104–107]. It was proposed that increasing the ratio of anti-apoptotic over pro-apoptotic Bcl-2-family members results in sensitization of IP₃R1 to basal IP₃ levels by a protein kinase A (PKA)-dependent phosphorylation of IP₃R1, thereby enhancing the basal Ca²⁺ leak through sensitized IP₃R1 channels and lowering the ER Ca²⁺ levels [108]. Other studies

proposed that Bcl-2 might directly impact the permeability of the ER membranes by mimicking ion channel functions [109–111]. Yet, the effect of Bcl-2 on ER Ca^{2+} store loading has not been confirmed in other studies [54,112–116]. The reason for these divergent observations could be related to the different cell models used. An elegant study of Foskett and co-workers showed that Bcl-Xl overexpression only lowers the ER Ca^{2+} -store content in DT-40 triple-IP₃R knockout cells ectopically expressing IP₃R3, but not in DT40 triple-IP₃R knockout cells or DT-40 triple-IP₃R knockout cells ectopically expressing IP₃R1 or IP₃R2 [112]. Thus, the eventual effect of anti-apoptotic Bcl-2 proteins on the steady-state ER Ca^{2+} levels may be dependent on the dominant IP₃R isoform expressed in the studied cell types. Alternatively, the effect of Bcl-2 proteins on the ER Ca^{2+} content might also be dependent on the presence of other proteins. For instance, lowering of the ER Ca^{2+} levels by Bcl-2/Bcl-Xl overexpression requires the presence of BI-1, since Bcl-2/Bcl-Xl overexpression fails to lower the ER Ca^{2+} levels in BI-1-deficient cells [117]. Thus, the effect of anti-apoptotic Bcl-2 proteins on the ER Ca^{2+} levels may also depend on the endogenous BI-1 expression levels in the studied cell types. BI-1 is a conserved pro-survival protein that acts as a Ca^{2+} /H⁺ antiporter [118], a Ca^{2+} channel [119] and an IP₃R sensitizer [65].

Beyond lowering of the ER Ca^{2+} -store content, Bcl-2 can directly target and differentially regulate IP₃R channels according to the spatiotemporal nature of the IP₃-induced Ca^{2+} release [115]. In T-lymphocytes, Bcl-2–IP₃R interaction enhances pro-survival Ca^{2+} oscillations induced by weak T-cell receptor activation while inhibiting pro-apoptotic transient Ca^{2+} elevation induced by strong T-cell receptor activity. It has also become clear that the anti-apoptotic Ca^{2+} effect of Bcl-2 is due to a direct interaction of its BH4 domain with the IP₃R, independently of its effect on the ER Ca^{2+} -store content [87,113–115,120,121] (Fig. 1). As such, recombinantly expressed and purified Bcl-2, or synthetic BH4-domain peptides, reduces IP₃R-single-channel activity in lipid bilayer experiments and IP₃R-mediated Ca^{2+} flux in permeabilized cells [113,114,116,122]. These functional observations were supported by molecular approaches, showing that Bcl-2 directly binds IP₃Rs. Purified Bcl-2 or BH4-domain peptides were shown to bind to the central, modulatory domain of the IP₃R. These studies also resulted in the identification of the Bcl-2-binding site on the IP₃R between residues 1389 and 1408. A 20 amino acid peptide covering these residues (named IP₃R-derived peptide (IDP)) was used as a decoy peptide that binds to Bcl-2 and disrupts the IP₃R/Bcl-2-protein complex. A TAT-fused version of stabilized IDP, generated by eliminating the predicted protease cleavage site (TAT-IDP^S), targets the BH4 domain of Bcl-2 and disrupts Bcl-2 binding to IP₃Rs, and consequently enhanced agonist-induced Ca^{2+} rise in a variety of cell types [123,124] (Fig. 1). It thereby potentiates Ca^{2+} -dependent apoptosis, e.g. in T-lymphocytes exposed to high [anti-CD3] without being cytotoxic by itself [113,114,123]. Interestingly, in B-cell malignancies, including CLL and DLBCL, but not in normal peripheral mononuclear blood cells, TAT-IDP^S causes spontaneous Ca^{2+} rises and triggers apoptotic cell death in the absence of any additional apoptotic stimulus [123,124]. Very recently, it has been reported that Bcl-2 interacts with dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) [125], an inhibitor of protein phosphatase 1 (PP1). DARPP-32 is activated by PKA-mediated phosphorylation at Thr34 and deactivated by CN-mediated dephosphorylation at this site [126]. These findings indicated that Bcl-2 docks DARPP-32 and CN on the IP₃R, creating a negative feedback loop. This protein complex responds to IP₃R-mediated Ca^{2+} release by inhibiting IP₃R phosphorylation at Ser1755, thereby suppressing excessive Ca^{2+} release capable of inducing cell death. Interestingly, this Bcl-2–CN–DARPP-32 feedback mechanism seems to be implicated in the regulation of Ser1755 phosphorylation and apoptosis in primary human CLL cells. Other anti-apoptotic Bcl-2-family members, including Bcl-Xl and Mcl-1, also interact with IP₃Rs and regulate IP₃R-mediated Ca^{2+} release [53,54,108,112,127,128]. Interestingly, Bcl-2, Mcl-1 and Bcl-Xl bind with comparable affinity to the carboxyl termini of all three mammalian

IP₃R isoforms, enhancing spontaneous IP₃R-dependent pro-survival Ca^{2+} oscillations and spiking in intact cells in the absence of agonist stimulation [54]. Moreover, in a recent study, the interaction between Bcl-Xl and VDAC has been shown to promote mitochondrial matrix Ca^{2+} accumulation by increasing Ca^{2+} transfer across the outer mitochondrial membrane, thereby enhancing mitochondrial bioenergetics and subsequent survival of the cells [129].

5. Bcl-2 interferes with the cell cycle

Although Bcl-2-family proteins are key players in the control of mitochondria-based apoptosis, they can also control cell proliferation (Fig. 2). High levels of Bcl-2 were reported to be associated with a lower proliferative capacity of human lymphoma [130,131], suggesting a negative control on proliferation. In primary murine T-lymphocytes, it was shown that Bcl-2 levels control the duration of the G₀ to S phase transition, with a critical point at the mid G₁ phase [4]. In bone marrow-derived BAF3 cells, Bcl-2 overexpression not only delays the onset of apoptosis, but also induces a rapid arrest in the G₁ phase of the cell cycle [132]. Moreover, thymocyte turnover is slower in transgenic mice overexpressing Bcl-2 than in the control mice. In this model system and upon in vitro stimulation with mitogens, B- and T-lymphocytes expressing the Bcl-2 transgene enter the cell cycle more slowly than control lymphocytes, while Bcl-2-deficient T-cells show increased cell-cycle progression [133]. It has also been reported that high Bcl-2 levels increase the doubling time of exponentially growing cells [134]. Moreover, Bcl-2 controls the expression pattern of cell-cycle regulators such as p130, p27 and pRb [4]. In general, the cell cycle control executed by Bcl-2 seems to be associated with its phosphorylation state. In cycling cells, the anti-apoptotic function of Bcl-2 is inactivated by JNK1 that becomes activated during the G₂/M phase, thereby phosphorylating Thr69, Ser70 and Ser87. These amino acids are located within the unstructured loop of Bcl-2 and were also phosphorylated in response to microtubule-damaging agents leading to the arrest of the cell cycle at G₂/M [135]. Moreover, mutating these JNK1-phosphorylation sites in Bcl-2 into alanines renders cells more resistant to apoptotic stimuli. As such, the phosphorylation of Bcl-2 at the G₂/M phase could be a way to eliminate altered or damaged cells, thereby avoiding their proliferation. Interestingly, JNK-mediated phosphorylation of Bcl-2 also seems to affect its ability to modulate ER Ca^{2+} homeostasis. Wild-type Bcl-2 and the phospho-dead Bcl-2^{Thr69Ala/Ser70Ala/Ser87Ala}, but not the phosphomimetic Bcl-2^{Thr69Glu/Ser70Glu/Ser87Glu} mutant, lowers steady-state ER Ca^{2+} levels [136]. Interestingly, the effect of Bcl-2 on the cell cycle seems to depend on its BH4-domain biology, since a mutation of a tyrosine residue (Tyr28) within the conserved N-terminal BH4 region of Bcl-2 to alanine (Tyr28Ala), serine (Tyr28Ser) or phenylalanine (Tyr28Phe), markedly reduces its ability to restrain re-entry of quiescent FDC-P1 myeloid cells or NIH 3T3 fibroblasts into the cell cycle upon exposure to growth factors [137]. This mutation does not affect the ability of Bcl-2 to heterodimerize with pro-apoptotic Bcl-2-family members, indicating that the role of Bcl-2 in the cell cycle is independent of Bcl-2's effect on Bax/Bak [137].

In T-cells, delayed cell cycle re-entry induced by Bcl-2 overexpression is associated with a reduction in IL-2, IL-3 or granulocyte-macrophage colony-stimulating factor (GM-CSF) expression. As also stated above, these growth factors are down-regulated due to an impaired NFAT activation [4]. The BH4 domain of Bcl-2 can sequester active CN and subsequently inhibit the NFAT-signaling pathway [37]. It could also reduce NFAT nuclear translocation due to its inhibitory effect on Ca^{2+} release from IP₃Rs [87,113–115,120,121]. As such, by reducing the Ca^{2+} release activity of the IP₃R, Bcl-2 could also exert an anti-proliferative role (Fig. 2). However, the addition of exogenous IL-2 only partially restores cell cycle progression, indicating that the Ca^{2+} –CN–NFAT pathway is not the only way Bcl-2 inhibits cell cycle progression [133].

6. Bcl-2 overexpression in cancer

Bcl-2 is highly expressed at the onset of many cancers [3,4,138]. In addition to B-cell lymphomas [139], Bcl-2 has been implicated in solid tumors like prostate cancer [140] and non-small cell lung cancer [141]. CLL and DLBCL are two examples of Bcl-2-overexpressing blood cancers [5,6]. One of the mechanisms of Bcl-2 upregulation in cancer cells is the translocation of the Bcl-2 encoding region of chromosome 18 to chromosome 14, downstream of the antibody heavy chain enhancer leading to its upregulation [142,143]. In addition, accumulating evidence indicates that overexpression of Bcl-2 can also occur via an alteration in microRNAs in CLL and DLBCL [144–149]. For instance, the microRNAs miR-15a and miR-16-1 that are frequently deleted in CLL, induce apoptosis by targeting Bcl-2 [144,148]. Moreover, in KPUM-MS3 and KPUM-UH1 cell lines derived from DLBCL patients, the overexpression of Bcl-2 correlates with the reduced expression of miR-143 and miR-145 [145].

Sustained proliferative signaling and resistance to cell death are two hallmarks of cancer development [150]. The common view is that cancer cells rely on high levels of Bcl-2 to counteract the ongoing upregulation of pro-apoptotic BH3-only proteins in response to oncogenic stress [32,151–153]. Bcl-2 neutralizes the activator BH3-only proteins, Bid and Bim, and the sensitizer BH3-only proteins, Bad, Puma, Bmf and to a lesser extent Bik [152] (Table 1 and Fig. 1).

In addition to its canonical anti-apoptotic role, Bcl-2 has been shown to have an inhibitory effect on the cell cycle and to induce cell quiescence [137] (Fig. 2). In cancer cells, the cell cycle control function of Bcl-2-family members can however be dissociated from the apoptosis control function [4]. Moreover, the inhibitory effect of Bcl-2 during cell cycle progression may contribute to the indolent nature of lymphomas highly expressing Bcl-2 [154]. Thus, the reported low tumor proliferation rate in non-Hodgkin's lymphoma may be a consequence of the inhibitory effect of Bcl-2 on cell cycle progression [130,131]. Moreover, patients whose breast cancer tissue shows high levels of Bcl-2 expression show a better prognosis [155]. In ovarian cancer cells, Bcl-2 delays cell cycle progression by promoting accumulation of cells in S phase without affecting the rate of apoptosis [156]. Because of their major role in the control of apoptosis, the impact of the cell cycle control function of Bcl-2 family members on tumor development is still not well defined. Eventually, the dual role of Bcl-2 in the enhancement of spontaneous IP₃R-dependent pro-survival Ca²⁺ oscillations in the absence of agonist stimulation [54,115] and in the protection from excessive IP₃-induced pro-apoptotic Ca²⁺ [87,113–115,120,121] may also contribute to the oncogenic nature of Bcl-2.

7. Bcl-2-based anti-cancer strategy: primed-to-death cells?

7.1. Primed-to-death at the mitochondria: BH3 mimetics

The concept of cancer cells being “primed-to-death” at the mitochondria was originally introduced by Letai and co-workers [157]. Priming-to-death at the mitochondria is related to the status of the Bcl-2 family members in the cell death program. In primed-to-death cancer cells, Bcl-2 is not free, but “loaded” with pro-apoptotic BH3-only proteins like Bim. Hence, cancer cells are addicted to high levels of Bcl-2 to neutralize their high levels of Bim, upregulated due to the ongoing oncogenic stress. Thus, antagonism of anti-apoptotic family members by sensitizer BH3-only domains or BH3-mimicking molecules only results in MOMP when the anti-apoptotic proteins are “primed” with activator BH3 proteins, as is the case in cancer cells but not in normal cells. Furthermore, the anti-apoptotic family members may be distinguished from each other based on their affinity for different BH3-only proteins and their isolated BH3 domain [157–159]. It is notable that the BH3 domains of activators Bid and Bim are bound to all anti-apoptotic Bcl-2 proteins, distinguishing them from the sensitizers, which show a more selective pattern of binding. While Bcl-2,

Bcl-Xl and Bcl-w proteins interact with the BH3 domain of Bad, Mcl-1 interacts with the two BH3 domains of Noxa, and Bfl-1 binds only PUMA [160]. The same group has developed an elegant technique called “BH3 profiling” to determine the cellular “addiction” to individual anti-apoptotic proteins [158]. For this, a panel of BH3-domain peptides derived from the different BH3-only proteins was used to selectively antagonize the individual anti-apoptotic Bcl-2-family members. Using purified mitochondria isolated from cancer cells, the cytochrome c release in response to BH3-domain peptides is assessed as a tool to identify blocks in apoptotic pathways in different cancer cells. Mitochondria of cancer cells lacking BH3-only proteins will undergo MOMP in response to BH3-domain peptides derived from activator BH3-only proteins but not of sensitizer BH3-only proteins. Mitochondria of cancer cells lacking Bax or Bak will be resistant to MOMP in response to BH3-domain peptides of both activator and sensitizer BH3-only proteins. Mitochondria of cancer cells requiring anti-apoptotic Bcl-2-family members to neutralize Bim will undergo MOMP in response to BH3-domain peptides of both activator and sensitizer BH3-only proteins. The latter class is indicated as being “primed-to-death”. Using BH3-domain peptides of different sensitizer BH3-only proteins, cancer cells’ “addiction” to a specific anti-apoptotic Bcl-2-family member can be determined. For instance, MOMP induced by Bad-BH3 peptide indicates that the cancer cells require Bcl-2, Bcl-Xl or Bcl-w. In contrast, MOMP induced by Noxa-BH3 peptide indicates that the cancer cells require Mcl-1. MOMP induced by PUMA, a promiscuous BH3-domain peptide targeting all anti-apoptotic Bcl-2-family members, but not by Bad or Noxa, indicates that the cancer cells require Bfl-1. Different experimental protocols have been established, allowing determining the “primed-to-death” status of cancer cells based on cytochrome c release from isolated mitochondria. This was done by using ELISA or based on the mitochondrial potential measured via lipophilic cation-1 (JC-1) in permeabilized cells [159].

It was shown that BH3 profiling and the amount of Bim scaffolded by anti-apoptotic Bcl-2 proteins can be used as a prediction for the apoptotic response of cancer cells to chemotherapy [21]. It is proposed that cancer cells, in which the mitochondria contain high levels of Bcl-2 and Bim, are typically closer to the “apoptotic threshold”. These cells are most sensitive to toxic stimuli, including chemotherapeutic drugs [153,161]. Hence from these recent studies, it seems that the mitochondrial apoptotic priming (determined by the response to the promiscuous PUMA peptide) can predict the tumor response to cytotoxic chemotherapy, the most common treatment used for human malignancies [153,161]. The mitochondrial priming state is thereby not the same in all malignancies [162]. For instance, cancers that are highly primed are those that respond most favorably to chemotherapy (blood cancers including CLL), whereas those that are unprimed respond poorly to chemotherapy (endometrial and renal cell carcinomas, serous borderline tumors) [21]. In the latter cases, the therapeutic window for using chemotherapy, and thereby killing cancer cells while saving normal cells, would be very limited.

Beyond chemotherapy, it is also clear that targeted induction of apoptosis and/or inhibition of anti-apoptotic proteins, particularly by antagonizing anti-apoptotic Bcl-2-family members, have become a novel therapeutic concept for cancer [163,164]. The experimental efforts using BH3 peptides have resulted in the development of BH3-mimetic drugs, like ABT-737 (from Abbott Laboratories) and its derivatives, including the orally available ABT-263. These BH3 mimetics function by slotting into the hydrophobic groove on the surface of Bcl-2 and Bcl-Xl, thereby blocking their capacity to inhibit apoptosis [165] (Fig. 1). Indeed, a correlation exists between the sensitivity of mitochondria to Bad-BH3 peptides and sensitivity of the cells to ABT-737 [151]. ABT-compounds have activity against multiple types of blood tumors [166] and some solid tumors [165,167,168]. Since Bcl-Xl activity is essential for platelet differentiation and survival [169], non-selective BH3 mimetic Bcl-2/Bcl-Xl antagonists result in thrombocytopenia and thrombocytopathy [170]. By comparing the effects of ABT-737 and its

analog ABT-263 on platelets and leukemia cells from the same donor, it has become clear that these Bcl-2/Bcl-XL inhibitors induce apoptosis at similar concentrations in leukemia cells and platelets [167,171]. These in vitro findings correlate well with a phase I study, which showed that thrombocytopenia was the main factor for the observed dose-limiting toxicity observed in CLL patients treated with ABT-263 [172].

Dysregulation of the intracellular Ca^{2+} homeostasis in platelets by ABT-737 has been reported to be implicated in this phenomenon, but this remains a controversial issue [171,173]. We have recently shown that unlike HA14-1, a non-specific Bcl-2 antagonist, which causes ER Ca^{2+} uptake inhibition in platelets and various human cell lines [174], ABT-737-induced thrombocytopenia is not caused by a dysregulation of intracellular Ca^{2+} homeostasis or dynamics (e.g. in response to thrombin) in platelets [175]. Thus, the observed dysregulation of Ca^{2+} signaling in ABT-737-treated platelets could be a consequence rather than being the cause of cell death.

A new BH3 mimetic with specific action on Bcl-2, ABT-199, has been developed and has no adverse effects on platelets but is still efficiently killing cancer cells [166]. It has been shown to be effective in non-Hodgkin's lymphoma, CLL, t(11; 14) multiple myeloma [176] and receptor-positive breast cancer [177]. Additionally, the BH3 mimetics may increase the sensitivity of tumors such as small-cell lung cancer to conventional chemotherapeutics, by removing blocks on the activation of apoptosis pathways [178]. In all cases, cancer cells are still able to develop resistance to any treatment however. Hence, it remains very relevant to find drug combinations, which could induce synergistic effects, preventing resistance development and overdose-related adverse effects.

There is crosstalk between the Bcl-2 family members and the pathways implicated in cell cycle progression, including mitogen-activated protein kinase (MAPK)–extracellular-signal-regulated kinases (ERK) and phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K)–Akt/PKB–PTEN. Both pathways are known to be classical drivers of G_1 to S phase progression [179,180]. In diverse cell types, activation of ERK1/2 signaling can lead either to the upregulation of pro-survival proteins of the Bcl-2 family, notably Bcl-2, Bcl-XL and Mcl-1, or to the decrease or inactivation of pro-apoptotic proteins such as Bim to achieve cell survival [181]. Bim expression is subjected to control both at the transcriptional and the post-translational levels [182] that are governed by various signaling pathways, including the ERK1/2 and PI3K–Akt/PKB pathways [181]. Interestingly, the knockdown of Bim can rescue HS2 leukemic cells from apoptosis induced by the MAPK–ERK pathway inhibitor, U0126 [183]. Moreover, the combination of Obatoclax, a non-specific Bcl-2 inhibitor to induce apoptosis, with NVP-BEZ235, a PI3K inhibitor to reduce cell proliferation, is required for a complete eradication of leukemic growth [183]. Hence, the combination of BH3 mimetics with cell cycle inhibitors likely will be instrumental for the treatment of leukemias.

Finally, it is important to note that BH3 mimetics (or other molecules that target the hydrophobic cleft of Bcl-2) might also affect signaling complexes at the ER. For instance, the molecular determinants responsible for Bcl-2, Bcl-XL and/or Mcl-1 binding to the C-terminal site of the IP_3Rs are not yet understood, but a contribution of the hydrophobic cleft should be considered [17]. Bcl-2 inhibitors, HA14-1 and BH3I-2', have been associated with IP_3R - and ryanodine receptor-dependent Ca^{2+} release in pancreatic acinar cells [184]. However, these compounds seem not to be specific for Bcl-2 and can induce cell death independently of Bax/Bak [185]. At least HA14-1 inhibited SERCA Ca^{2+} ATPase and caused intracellular Ca^{2+} dysregulation [175]. In any case, specific Bcl-2 antagonism using ABT-737 did not trigger Ca^{2+} -signaling events in primary CLL cells [123]. Besides the potential effects of BH3 mimetics on the IP_3R channels, they can also affect IRE1 α activation during prolonged ER stress [100]. BH3-only proteins (like Bim and Puma) have been implicated in establishing sustained IRE1 α signaling in cells exposed to prolonged ER stress. Interestingly, the formation of complexes between these BH3-only proteins and Bcl-2 (but not Bcl-XL

or Mcl-1) seems to be required for sustained IRE1 α signaling and promoting an early adaptive response by upregulating UPR genes via X-box binding protein 1 (XBP-1) splicing (XBP1-s). The kinetic profile of these adaptive UPR responses clearly precedes pro-apoptotic BH3-only functions at the mitochondria. BH3 mimetics, like ABT-737, and Bad prevent this sustained IRE1 α signaling by disrupting the Bcl-2/Bim complexes. Hence, it is clear that BH3-mimetic compounds may negatively impact the survival of (cancer) cells by abrogating sustained IRE1 α activation needed for engaging an adaptive UPR program.

7.2. Primed-to-death at the ER: BH4-domain-targeting peptides

Given Bcl-2's critical role at the ER where it suppresses toxic Ca^{2+} -signaling events, we anticipate that cancer cells might be addicted to Bcl-2 at the ER Ca^{2+} stores for their survival, likely via the BH4-domain biology of Bcl-2. Indeed, exposing primary CLL cells or DLBCL cell lines to TAT-IDP^S, a peptide that targets the BH4 domain of Bcl-2, results in spontaneous pro-apoptotic Ca^{2+} spikes and mitochondrial Ca^{2+} overload, eventually leading to apoptotic cell death [123,124]. In a set of DLBCL cell lines, we found a heterogeneous response to TAT-IDP^S treatment. Strikingly, TAT-IDP^S-induced Ca^{2+} rise in the cytosol and TAT-IDP^S-induced apoptosis correlate with the expression level of $\text{IP}_3\text{R2}$ levels but neither with the total IP_3R levels nor with the levels of $\text{IP}_3\text{R1}$ or $\text{IP}_3\text{R3}$ channels. Interestingly, $\text{IP}_3\text{R2}$ is the IP_3R isoform with the highest sensitivity to its ligand IP_3 [186]. Thus, high $\text{IP}_3\text{R2}$ levels may render cancer cells vulnerable to basal IP_3 concentrations, thereby leading to mitochondrial Ca^{2+} overload and MOMP [11], and/or depletion of the ER Ca^{2+} stores, leading to chronic ER stress and apoptosis [187,188]. In particular, basal IP_3 signaling might be elevated in CLL and DLBCL cells, since they display chronic B-cell-receptor signaling [189,190]. As such, the presence of the $\text{IP}_3\text{R2}$ hypersensitive channels may be one of the factors that contribute to cancer cells being “primed-to-death” at the level of the ER through a mechanism that drives Ca^{2+} -mediated apoptosis. In contrast, TAT-IDP^S-resistant DLBCL cells mainly express $\text{IP}_3\text{R3}$, although there is no such striking correlation as with TAT-IDP^S-sensitivity and $\text{IP}_3\text{R2}$ expression [124]. The IP_3 levels in chronically activated B-cells may not be sufficient to trigger activation of $\text{IP}_3\text{R3}$, which is the least sensitive IP_3R isoform. As a result, cells expressing $\text{IP}_3\text{R3}$ may not require Bcl-2 at the ER, given the low sensitivity of this isoform to basal IP_3 signaling. This concept is supported by co-immunoprecipitation experiments using cell lysates from TAT-IDP^S-sensitive and -resistant DLBCL cell lines [124]. These experiments showed that the fraction of the total amount of Bcl-2 that co-immunoprecipitates with $\text{IP}_3\text{R2}$ in TAT-IDP^S-sensitive DLBCL cells is much larger than the fraction of total Bcl-2 that co-immunoprecipitates with $\text{IP}_3\text{R3}$ in TAT-IDP^S-resistant DLBCL cells. It is important to note that in surface plasmon resonance-binding experiments in vitro, all IP_3R isoforms are capable to interact with the BH4 domain of Bcl-2 [116]. Additional parameters affect the mechanism of IP_3R regulation by Bcl-2, which could differ for the particular isoform. For instance, recent reports showed that Akt/PKB, often hyper-activated in DLBCL cells [191], selectively suppresses Ca^{2+} flux through $\text{IP}_3\text{R3}$ subtype channels [61,62]. More recently, the tumor suppressor PTEN was reported to be located in the MAMs and to counteract Akt/PKB kinase-mediated reduction in Ca^{2+} release via IP_3Rs by exerting a protein phosphatase activity [63].

The mechanisms that control $\text{IP}_3\text{R2}$ (over-)expression in these cancer cells are not fully understood. Also, the advantage of cancer cells expressing high levels of potentially “toxic” channels such as the $\text{IP}_3\text{R2}$ remains to be investigated. It is remarkable that endogenous $\text{IP}_3\text{R2}$ levels are rather low in most tissues. However, $\text{IP}_3\text{R2}$ channels seem highly expressed in organs with high metabolism, such as organs with exocrine functions [192]. Yet, one aspect may involve the role of IP_3R channels and specific IP_3R isoform in controlling cancer cell metabolism. In acinar cells, $\text{IP}_3\text{R2}$ expression levels have been linked to the sensitivity toward metabolic stress, as $\text{IP}_3\text{R2}$ is the most sensitive

isoform toward ATP regulation and determines the influence of ATP depletion on intracellular Ca^{2+} signaling [193]. Since ATP is a positive regulator of IP_3R activity [193,194], a decline in cellular ATP levels, e.g. during metabolic stress in cancer cells [195], may therefore compromise IP_3R -mediated Ca^{2+} signaling necessary for mitochondrial bioenergetics and their survival. As a consequence, these cells might compensate this weakened IP_3R -mediated Ca^{2+} signaling by increasing basal IP_3 signaling and by upregulating an IP_3R isoform that is more sensitive to IP_3 .

To further scrutinize the concept that $\text{IP}_3\text{R}2$ is a factor that contributes to the “addiction” of cancer cells to Bcl-2 at the ER and being primed-to-death at the level of the ER, we performed a search of the GeneSapiens microarray database website (www.genesapiens.org). We screened for the expression levels of $\text{IP}_3\text{R}2$ mRNA across 9783 samples from 175 different types of healthy and diseased tissue samples, including different tumors [196,197] (Fig. 3). Interestingly, this analysis shows a high $\text{IP}_3\text{R}2$ mRNA level in samples from CLL patients. The CLL B-lymphocytic blast cells are also characterized by a chronic B-cell-receptor signaling [190]. In fact, this correlates with previous findings [123], showing that primary cells obtained from peripheral blood of CLL patients are very sensitive to BH4-domain targeting peptides that disrupt Bcl-2– IP_3R interaction resulting in Ca^{2+} -driven apoptosis.

7.3. Opposite sensitivity to BH3-mimetic drugs versus BH4-domain targeting compounds?

Finally and most interestingly, from the comparison between our analysis in DLBCL [124] and the results obtained recently by Souers and co-workers [166], we found that SU-DHL-4 cells that are very sensitive to TAT-IDP^S displayed a low sensitivity to the BH3-mimetic drugs, ABT-263 (Navitoclax; Abbott Laboratories, the orally bioavailable version of ABT-737) and ABT-199 (high EC_{50} values of $\sim 23.3 \mu\text{M}$ and $7.1 \mu\text{M}$, respectively, as shown in [166]) (Fig. 4). On the other hand,

OCI-LY-1 cells that are very resistant to TAT-IDP^S display a very high sensitivity to BH3-mimetic drugs (low EC_{50} values of $\sim 0.5 \mu\text{M}$ and $0.02 \mu\text{M}$, respectively, as shown in [166]). Hence, it seems that there is an opposite response to BH4-domain-targeting tools and BH3-mimetic tools that target the hydrophobic cleft. It is tempting to speculate that cancer cells addicted to Bcl-2 at the mitochondria to prevent Bim-dependent Bax activation, will be particularly sensitive to BH3-mimetic drugs, while cancer cells addicted to Bcl-2 at the ER to prevent toxic IP_3 -induced Ca^{2+} signaling through $\text{IP}_3\text{R}2$ channels will be particularly sensitive to BH4-domain targeting tools (like TAT-IDP^S). Clearly, the concept that cancer cells might be addicted to Bcl-2 at two different compartments, the mitochondria and the ER, thereby dictating their apoptotic response to BH3 mimetics and BH4-domain-targeting tools, ought to be further scrutinized.

8. Conclusion

We propose that the anti-apoptotic function of Bcl-2 in cells occurs at least at two levels, both at the mitochondria where Bcl-2 prevents Bim-mediated Bax/Bak activation, and at the ER where Bcl-2 prevents toxic Ca^{2+} release through IP_3Rs . The “addiction” of cancer cells to high levels of Bcl-2 may depend on the oncogenic mechanisms that may upregulate either Bim or $\text{IP}_3\text{R}2$ channels. Interestingly, different Bcl-2-protein domains seem to be responsible for its action at the mitochondria versus the ER. The hydrophobic cleft of anti-apoptotic Bcl-2 scaffolds and neutralizes pro-apoptotic BH3-only proteins and Bax/Bak at the mitochondria. As such, cancer cells requiring Bcl-2 at the mitochondria will be very sensitive to BH3-mimetic drugs. On the other hand, the N-terminal BH4 domain of anti-apoptotic Bcl-2 binds and inhibits pro-apoptotic Ca^{2+} flux through IP_3Rs at the ER. As such, cancer cells requiring Bcl-2 at the ER will be very sensitive to BH4-domain targeting drugs. The field has significantly advanced in understanding the mechanisms that underlie mitochondrial priming, exploiting these insights with novel therapeutic drugs, and linking them to the clinical

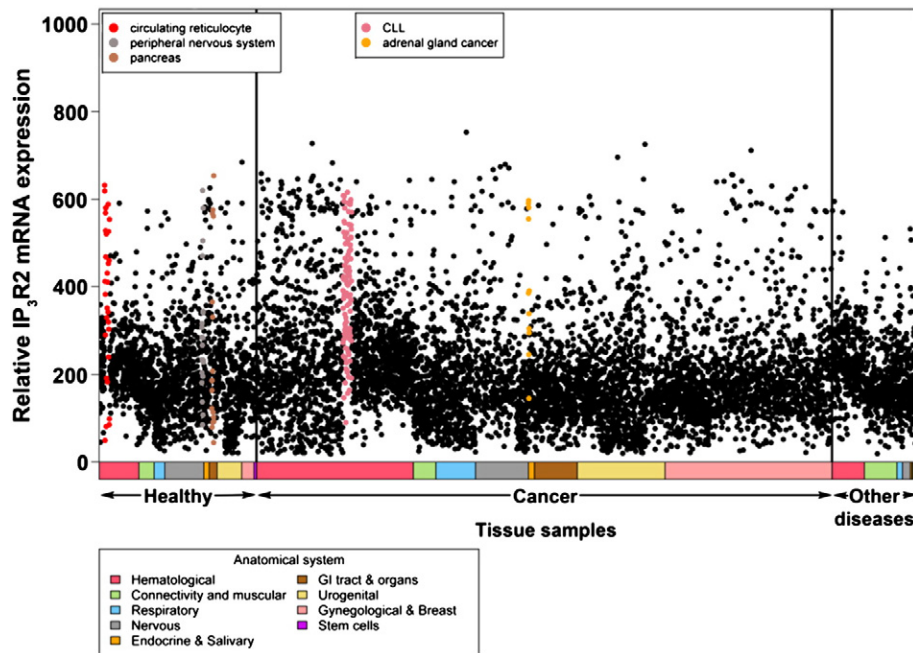


Fig. 3. Detailed gene expression profile of $\text{IP}_3\text{R}2$ across the GeneSapiens microarray database (www.genesapiens.org). A bioinformatics method for the comparison of mRNA expression levels of human genes across 9,783 Affymetrix gene expression array experiments representing 43 distinct healthy tissue types, 68 distinct malignant tissue types and 64 distinct non-tumor diseases. 70% of the samples are from malignant tumors, 19% are from healthy tissue and 11% are from other diseases. In this GeneSapiens plot, all the 9,783 samples are represented along the x-axis in a predetermined order, first the normal tissues, then cancers and then other diseases. The y-axis reflects the relative level of $\text{IP}_3\text{R}2$ gene expression after the three-step in silico normalization approach previously described in [196]. Each dot represents the relative expression of $\text{IP}_3\text{R}2$ in one sample. Anatomical origins of each sample are marked with colored bars below the gene plot. Sample types having higher-than-average expression or an outlier expression profile are additionally colored in the figure (legend at the top left corner). This analysis shows an outlier expression profile of $\text{IP}_3\text{R}2$ mRNA in chronic lymphocytic leukemia (CLL) and adrenal gland cancer. The levels of $\text{IP}_3\text{R}2$ mRNA are normally high in healthy circulating reticulocytes, in the peripheral nervous system and in the pancreas. $\text{IP}_3\text{R}2$ is the gene name of $\text{IP}_3\text{R}2$ (used in the GeneSapiens microarray database but not present in the figure).

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